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Hepatitis B Antigen and Systemic Lupus Erythematosus*

False Positive Complement Fixation Due to Anti-antibodies

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Hepatitis B Antigen und Lupus erythematodes disseminatus

Falsch positive Komplementbindung durch Anti-Antikörper

Zusammenfassung. Seren von 22 Patienten mit Lupus erythematodes disseminatus (LED) wurden auf das Vorliegen des Hepatitis-B-Antigens (HB_sAg) untersucht. Zu diesem Zwecke wurden 3 verschiedene Methoden eingesetzt: 1. Komplement-Fixationstest (KFT), 2. Überwanderungselektrophorese (UEP) und 3. Radio-Immunoassay.

Seren von 8 Patienten zeigten positive Resultate im KFT. Dieselben Seren und solche von 28 weiteren LED Patienten enthielten kein HB_sAg, wenn sie mit UEP und Radio-Immunoassay getestet wurden.

Zusätzliche Studien dienten der Charakterisierung des für den positiven KFT verantwortlichen Faktors. Es zeigte sich, daß die LED Seren auch Komplement in Gegenwart von normalen Seren fixierten, wenn letztere mehrfach gefroren und aufgetaut oder auf 65° C erhitzt worden waren. Der Komplement-fixierende Faktor war absorbierbar mit aggregierten Gammaglobulinen, nicht aber mit HB_sAg. Die Komplement fixierende Aktivität des Faktors wurde durch Behandlung der Seren mit 2-Merkapto-Aethanol abgeschwächt.

Die falsch positiven Komplement-Fixationsteste mit LED Seren und anti HB_sAg Antiseren können demzufolge auf das Vorliegen von Anti-Antikörpern mit Gammaglobulinspezifität zurückgeführt werden.

Schlüsselwörter: Hepatitis B-Antigen – Lupus erythematodes disseminatus – Komplementfixation.

Summary. Sera from 22 patients with systemic lupus erythematosus (SLE) were examined for the presence of hepatitis B antigen (HBsAg) by a complement fixation (CF) test, by an immunoelectrophoretic method (counterelectrophoresis-CEP), and by radioimmunoassay (RIA). The sera from 8 patients gave positive results using CF. However, the same sera and sera from 28 additional SLE patients, when tested with CEP and RIA, were not shown to contain HBsAg.

Additional studies were carried out in order to characterize the factor responsible for the false positive CF of the 8 SLE sera. It was shown that the sera also fixed complement in presence of normal serum previously submitted to freezing and thawing or heating at 65°C. The complement fixing factor was readily absorbed by aggregated IgG but not by insolubilized HBsAg. Complement fixation was strongly diminished by 2-mercaptoethanol treatment of SLE serum. It thus appears that the false positive reactions for the presence of HBsAg obtained by CF are due to the occurrence of "anti-antibodies" reacting with aggregated IgG. There is no increased incidence of HBsAg in the serum of SLE patients.

Key words: Hepatitis B antigen – Systemic lupus erythematosus – Complement fixation.

Introduction

Viruses have long been suspected as important causative factors in human SLE and in the occurrence of antinuclear antibodies seen in some strains of mice [7]. In man, antibodies to myxo- and paramyxoviruses have been found in systemic lupus erythematosus [4]. Furthermore, a lupus-like syndrome may accompany chronic active hepatitis, a disease which is known for its association with viruses, especially with hepatitis B virus antigen (HBsAg). Therefore, HBsAg has been searched for intensively by a variety of methods in the sera of patients with SLE and with other con-

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nective tissue diseases. While some authors found HBsAg using microcomplement fixation [1] and sandwich counterelectrophoresis [19], other studies using RIA, CEP or hemagglutination techniques failed to demonstrate HBsAg in SLE [10, 13, 14, 16].

The present study was designed in order to investigate more precisely the problem of HBsAg in SLE using three different methods, i.e. the RIA technique using guinea pig antibody (Abbott), the CEP and a recently described semi-automated complement fixation technique (CFT) which allows for a direct calculation of the specific CF.

Since some SLE sera were found to fix complement in presence of anti-HBsAg antibody, but failed to demonstrate HBsAg by the other two procedures, we have carried out additional studies to try to explain the positive results with CFT.

Material and Methods

50 patients suffering from SLE, 5 patients suffering from rheumatoid arthritis (RA), 12 clinically healthy blood donors carrying the HBsAg and 40 normal blood donors were included in this study. Standard clinical, biological and histological criteria were used for the diagnosis of SLE [3]. Disease activity was judged by the following criteria: nephritis, blood urea nitrogen, hemolytic complement activity and DNA binding activity. Blood was only collected from patients with active disease. Diagnosis of RA was based on classical criteria according to the standards established by the American Rheumatism Association [12]. The latex agglutination titers of these seropositive cases varied from 1/80 to 1/2560.

Blood was allowed to clot at room temperature for 60 min. Serum was separated by centrifugation at 4°. It was then used after heat inactivation (30 min 56° C).

Detection Methods for HBsAg

The CEP was used to test all 122 serum specimens included in this study and was performed according to ref. [11]. The AUSRIA® 125 assay for HBsAg (Abbott Laboratories, North Chicago, Ill., USA) was used to test all 80 serum specimens from 50 patients with SLE and all sera from the 15 patients with RA.

The CFT which was used is based on the general principles of this technique for the detection of HBsAg [15]. We have recently described a continuous flow system for dosage of hemolytic complement [8] which was further adapted to a semiautomated technique for quantitative evaluation of specific CF [9]. Sheep erythrocytes (Institut Mérieux, Lyon, France), sensitization with amboceptor (Wellcome Research Laboratories, Beckenham, England) to give freshly sensitized erythrocytes (EA) as well as buffers and flow design were the same as described earlier [8]. For the present study serum from guinea pigs (Institut Pasteur, Paris, France) was serially diluted and its hemolytic activity tested on the machine with an EA suspension containing 9×10^7 EA/ml of buffer. The CF protocols consisted of incubation of 0.4 ml antibody dilution, 0.5 ml guinea pig complement (GP-C) and 0.4 ml of antigen (patient's serum) which were mixed in this order. The final GP-C dilution was the same as that which revealed 90% EA lysis when HBsAg and anti-HBsAg antibody were replaced by buffer, i.e., in most cases, 1:300. The appropriate anti-HBsAg antibody dilution was assessed in checkerboard titrations and was found to be 1/10. Anti-HBsAg antisera were purchased from Biotest, Frankfurt/M., Germany. Two other antisera were obtained from a multitransfused hemophiliac who was shown to carry the HBsAg and its antibody on the basis of CEP studies. These antisera did not contain DNA or anti-DNA as determined by a radioimmunoassay [18].

All sera were tested after heat inactivation and assayed in serial dilutions ranging from 1/2 to 1/2048. Each dilution was also assayed in the absence of anti-HBsAg antiserum in order to determine the anticomplementary activity of the patient's serum *per se*. Anti-HBsAg antibody as an additional control was also assessed in the absence of patient's serum. Before testing in the continuous flow system, the mixtures were incubated 90 min at 37° C and 12 hours at 4° C. Residual hemolytic complement activity was tested on the Auto Analyzer (Technicon, Technical Dept.) and compared to a reference curve established with the serial GP-C dilutions, which were incubated under the same conditions but without HBsAg and its antibody. For all experiments, the Technicon Sampler IV was fitted with a 40 samples/hour cam at a sampling/wash ratio of 1/1. The sum of antibody-CF and patient's serum CF was subtracted from the overall serum-antiserum CF resulting in a difference which we call specific CF.

Other Immunological Techniques

Anti-DNA antibodies were assessed according to Wold [18]. For these tests, DNA was prepared by cultivation of human fibroblast cells in the presence of tritiated thymidine. The tritiated DNA (3H-DNA) was extracted and purified according to Marmur's technique [6].

IgG fractions from SLE sera were obtained by chromatography on DEAE cellulose according to routine laboratory procedures. The rheumatoid factor activity of sera was measured by the slide latex fixation test (Hyland Laboratories, Los Angeles, USA). Human IgG was prepared according to Kistler and Nitschmann [5] and supplied by the Central Laboratory of the Swiss Red Cross (Berne, Switzerland). It contained IgG, IgA, and IgM at a ratio of 600:7:1. Heat aggregation was performed for 20 min at 60° C and the aggregates (aggHGG) were used fresh. Absorption of SLE sera and IgG fractions thereof was done with HGG which had been insolubilized using ethylchloroformiate according to Avrameas [2]. As a rule, the ratio between the SLE-IgG and the insolublized aggHGG was 1/20. Absorption took place at 37° C for 30 min. The same technique was used for absorption of anti-HBsAg with insolublized HBsAg.

For reduction and alkylation, 0.2 ml of sera from patients with SLE or RA were incubated with 0.4 ml of 2-mercaptoethanol at 37° for 60 min. Thereafter, 0.2 ml of 0.66 M iodoacetamide were added. Incubation took place at 4° for 60 min. Control sera were only incubated with 0.4 ml buffer. All mixtures were dialysed for 24 h against VBS before testing the anticomplementary activity in presence of GP-C. For control of the efficiency of the reduction and alkylation procedure, 0.2 ml NHS containing aggHGG (1 mg/ml) were also treated with 2-ME and iodoacetamide and it was seen that their anticomplementary effect in presence of GP-C was completely abolished.

Results

The results of HBsAg testing obtained with the three different methods are listed in Table 1. The sera of 8 out of 22 patients suffering from SLE fixed comple-

Table 1. Detection of HBsAg by three different methods in various disease states

Diagnosis	No. of patients	Method		
		Complement fixation	Counter electrophoresis	Radioimmune assay
Systemic lupus	22	8 (36%)	0	0
	28	ND[a]	0	0
Rheumatoid arthritis	6	6	0	0
HBsAg carriers (blood donors)	12	12	12	12
Healthy blood donors	40	0	0	0

[a] ND = not done.

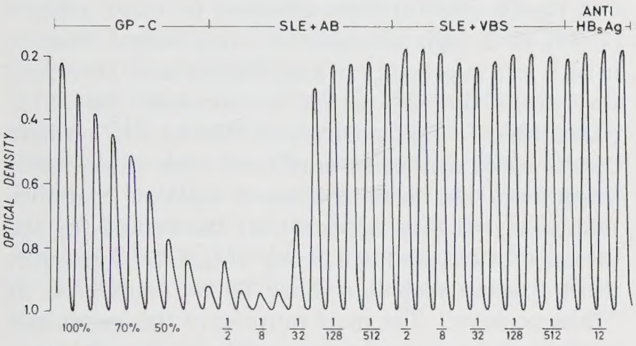

Fig. 1. Trace writing of one typical experiment. From left to right: the first block of peaks are dilutions of the GP-C for establishment of a reference curve. The next block of 10 peaks represents the CF by mixture of serial dilutions of an SLE serum with constant amounts of anti-HBsAg antiserum. Maximal CF occurs at SLE serum dilution of 1/8. Note pro- and postzone phenomenon. Third block of peaks: SLE serum dilutions in presence of GP-C alone. Last 3 peaks: antiserum in presence of GP-C alone

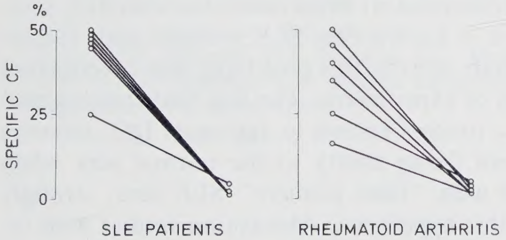

Fig. 2. Abolition of specific CF by SLE and RA sera after treatment with 2-mercaptoethanol. The sera were incubated with freeze and thawed normal human serum in presence of GP-C. The results of the experiments performed without reduction and alkylation are plotted near the ordinate. The CF by each individual serum after treatment is situated near the zero point. The values obtained on each serum before and after treatment are linked by a straight line

ment when incubated with anti-HBsAg serum. The trace writing of a typical experiment is set out on Fig. 1. For this SLE patient, maximum complement fixation can be seen at a dilution of 1/8; lower or higher dilutions of the patient's serum reveal less complement fixation (zone phenomenon). After subtraction of individual complement fixation values of patient's serum alone, and anti-HBsAg alone, the specific complement fixation for this particular patient was 73%. It should be mentioned that not all anti-HBsAg antisera were suitable for such studies since some batch numbers exhibited a strong intrinsic complement fixation. Such antisera were forthwith excluded from this study.

None of the 8 SLE sera which fixed complement upon incubation with anti-HBsAg serum revealed detectable amounts of HBsAg when tested with CEP and RIA. Moreover, in sera from 28 other SLE patients, the HBsAg was not detectable when tested by CEP and RIA. The latex fixation test for rheumatoid factor activity revealed titers below 1:4 in all of the 8 SLE sera with a positive complement fixation. The serum of 6 patients with rheumatoid arthritis also fixed complement when incubated with the anti-HBsAg antiserum although to a lesser extent than the SLE sera (Fig. 2).

Additional studies were carried out on the sera of the 8 SLE patients who exhibited a positive complement fixation test for HBsAg.

Role of the Anti-HBsAg Antiserum

Complement fixation tests were performed with 5 different anti-HBsAg antisera in presence of the 8 SLE sera: 4 antisera which had been frozen before using them for this purpose, and 1 fresh antiserum from a multi-transfused hemophiliac patient. Positive complement fixation results were obtained with the 4 antisera which had been stored frozen from 2 to 6 months. The amount of complement fixed varied from serum to serum. No complement fixation was obtained with the fresh anti-HBsAg serum.

Complement Fixation tests with Incubation Mixtures of SLE Serum and Normal Serum

When the 8 SLE sera were incubated individually with fresh normal serum from 10 donors no significant amounts of complement were fixed. Normal human sera were then used after they had been repeatedly frozen and thawed (2×, 4×, 8×, 16× and 32×). When the sera had been frozen and thawed more than 8 times they became highly anticomple-

mentary in presence of the SLE sera. When normal sera freeze-thawed 2 to 8 times were assayed with the SLE sera, varying amounts of complement were fixed by the reaction mixture. Reproducible results were obtained with normal sera which had been frozen and thawed 8 times and used in a dilution of 1:16 (i.e., in a dilution which no longer proved anticomplementary *per se*). Under these conditions, the mean complement fixation of the 8 SLE sera was 40% with a standard deviation of 6%.

For heat aggregation, a normal serum was incubated at 65°C for 20 min, and serially diluted for assaying the anticomplementary activity. At a dilution of 1:64, the anticomplementary activity produced a drop of 6% in hemolytic activity of the added GP-C. When assayed at a 1:64 dilution with the 8 SLE sera a specific CF of 44%+8% (±1 SD) was obtained.

In all studies normal sera were used instead of SLE sera to control the specificity of the results obtained with SLE sera. Under all the above-mentioned conditions normal serum did not lead to complement fixation when incubated with the treated sera.

Absorption Experiments

From the results obtained so far, the antigenic substrate responsible for complement fixation does not seem to be HBsAg. In order to assess the nature of the reactant substrate, absorption experiments were carried out with insolublized HBsAg and with aggregated IgG. The results of these experiments are summarized in Table 2. In addition to SLE sera, their IgG fractions obtained by chromatography on DEAE cellulose were also assayed, absorbed or not by aggregated IgG and by HBsAg. The results obtained with IgG fractions from 4 different SLE sera were similar to those obtained with the whole SLE sera. When sera containing anti-HBsAg antibody were absorbed with insolublized HBsAg, their complement fixation became negative (Table 2).

Six sera from the 8 SLE patients and 6 sera from RA patients were submitted to mercaptoethanol treatment and subsequent alkylation with iodoacetamide. The sera were reacted before and after this treatment with 1:8 diluted normal serum which had been frozen and thawed 8 times in presence of GP-C. Complement fixation was completely abolished by this treatment (Fig. 2).

Table 2. Specific CF before and after absorption of test serum and/or antiserum

Test serum	No. of sera tested	Antiserum		*T* test
		Anti-HBsAg	Anti-HBsAg absorbed[a]	(*p* value)
SLE sera:				
native	8	39±9	33±12	>0.5
absorbed[a]	8	4±4	4±4	>0.5
IgG from SLE sera:				
native	4	34±6	33±3	>0.5
absorbed[b]	4	2±2	3±3	>0.5
Controls:				
NHS	8	2±2	1±1	>0.5
sera containing HBsAg	8	52±32	2±2	<0.0002

[a] Absorbed by insolublized HBsAg.
[b] Absorbed by aggHGG.

Discussion

Our results confirm those obtained by other authors [1, 19] of a high incidence of complement fixation in SLE sera in presence of anti-HBsAg sera. However, the results do not permit to conclude that SLE patients have a high incidence of HBsAg. The positive results could neither be confirmed with counterelectrophoresis nor with the most sensitive radioimmunoassay. It thus appears that the results for the presence of circulating HBsAg obtained by complement fixation technique have to be considered as "false positive". The main purpose of this paper was to study the mechanism responsible for the false positive reaction.

At first, it was possible to rule out DNA-anti DNA reactions as being responsible for complement fixation by the observation that the anti-HBsAg sera did not contain sufficient amounts of DNA to fix complement with SLE sera containing high DNA binding activity. The second possibility was a reaction involving serum iso-antibodies. This possibility was excluded by the fact that none of 10 normal fresh sera fixed complement when incubated with SLE sera.

Since it is known that SLE patients may exhibit anti-antibody activity this possibility was investigated by a series of experiments. The fact that freezing and thawing, a process known to aggregate IgG, conveys complement fixing ability to the normal sera when incubated with "false positive" SLE sera, strongly supports this hypothesis. Absorption studies with insolubilized HBsAg and heat aggregated IgG further support this possibility, since only aggregated gammaglobulin and not insolubilized HBsAg rendered the SLE sera negative with regard to complement fixation in presence of anti-HBsAg antisera. Finally, it appears that the reason the normal sera fix complement in presence of some SLE sera is due to the formation

of aggregated IgG and not because they contained antibodies to HBsAg.

The question was then to find out whether the responsible factor is a gamma-globulin. Indeed, the IgG fraction obtained from false positive SLE sera, when incubated with sera containing aggregated IgG, exhibited a capacity to fix complement similar to that of the native SLE serum. This capacity furthermore was abolished upon absorption with aggregated IgG.

It was therefore important to investigate serum containing the rheumatoid factor, an IgM antibody to aggregated IgG, in the complement fixation assay. The results were rather similar to those obtained with the 8 SLE patients. Reduction and alkylation of both SLE and RA sera resulted in a complete abolition of the complement fixing capacity in presence of aggregated IgG, although for somewhat different reasons. While the IgM rheumatoid factor is completely destroyed by this procedure, an IgG antibody is only slightly modified. However, as previously shown this treatment greatly reduced the capacity of IgG antibodies to fix complement [17].

In conclusion, one third of SLE patients have been shown to fix complement when assayed with "old" anti-HBsAg antisera. This reaction has been shown to be due to IgG anti-aggregated IgG antibodies and not to the presence of the HBsAg in the SLE sera. The incidence of HBsAg in patients with SLE does not appear to be greater than in the general population.

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